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Covariations in the nuclear chloroplast transcriptome reveal a regulatory master-switch

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The evolution of the endosymbiotic progenitor into the chloroplast organelle was associated with the transfer of numerous chloroplast genes into the nucleus. Hence, inter-organellar signalling, and the co-ordinated expression of sets of nuclear genes, was set up to control the metabolic and developmental status of the chloroplast. Here, we show by the differential-expression analysis of 3,292 genes, that most of the 35 environmental and genetic conditions tested, including plastid signalling mutations, elicit only three main classes of response from the nuclear chloroplast transcriptome. Two classes, probably involving GUN (genomes uncoupled)-type plastid signalling, are characterized by alterations, in opposite directions, in the expression of largely overlapping sets of genes.

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INTRODUCTION

Almost all of the ~3,000 higher-plant chloroplast proteins are nucleus-encoded (Abdallah *et al.*, 2000; Leister, 2003). The adaptation of the chloroplast to fluctuations in the environment is reflected by the expression levels of corresponding nuclear genes (the nuclear chloroplast transcriptome; Desprez *et al*., 1998; Kurth *et al*., 2002). Gene expression is modulated by plastid-to-nucleus signalling, which is controlled by the stromal ATP:ADP ratio, the transthylakoidal pH gradient, and by the redox state of photosynthetic components and other molecules (Pfannschmidt *et al*., 2001). The analysis of mutants that express light-regulated genes in the absence of chloroplast development (Susek *et al*., 1993; Mochizuki *et al*., 2001; Surpin *et al*., 2002) has identified another type of plastid-to-nucleus signalling: all known 'genomes uncoupled' (*gun*) mutants are defective in in tetrapyrrole biosynthesis.

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The analysis of messenger RNA expression using DNA arrays allows the study, on a genomic scale, of how the organellar state modulates gene transcription (Kurth *et al*., 2002; Legen *et al*., 2002). We report the characterization of the nuclear chloroplast transcriptome under 35 environmental and genetic conditions, which allowed us to predict the existence of a general mechanism for the transcription-based regulation of organelle function. The results of our analysis suggest the presence of a transcriptional switch that functions in a binary mode, by either inducing or repressing the same large set of nuclear genes that are relevant to plastid functions.

RESULTS

A chloroplast gene-sequence-tag array for 3,292 genes

The scanning of the complete genome sequence of *Arabidopsis thaliana* for chloroplast transit peptides (cTPs) identified 2,661 proteins that are likely to be targeted to the chloroplast. For ~75% of the corresponding open reading frames (ORFs), gene expression has been confirmed by expressed-sequence-tag (EST) sequencing. To analyse whether the remaining ORFs are also transcribed, we studied their expression under various inducing conditions. Gene-sequence tags (GSTs) for the 2,661 nuclear chloroplast genes, and for 631 genes encoding non-chloroplast proteins, were amplified by PCR and spotted onto nylon membranes to generate a 3,292-GST array.

Wild-type plants, and several mutants with defects in chloroplast function and/or plastid-to-nucleus signalling, were grown under various conditions; in all, 35 conditions were tested (Table 1). For all 3,292 genes, a significant change in expression was detected in at least one genetic or environmental context, indicating that the corresponding ORFs are transcribed and, furthermore, that their constitutive expression under all conditions is unusual.

Responses of the nuclear chloroplast transcriptome

The 35 conditions tested showed substantial variation in their ability to elicit a transcriptional response; 15–76% of the 3,292 genes showed a significant differential response in photosynthetic tissue (Fig. 1). With respect to the ratio of upregulated to downregulated genes, three main types of transcriptome response were identified: two types of response were predominantly associated with either upregulation or with downregulation. A third type of response involved approximately equal numbers of upregulated and downregulated genes. Among the 35

Table 1 | Overview of conditions used for expression profiling

Abbreviation	Genotype or treatment	Main effects on chloroplast function
Treatments		
PSII/I	Growth under PSII-specific light versus PSI-specific light*	Photosynthesis; plastid redox state
L 30′	30 min light, versus darkness	Various functions
HL 15′	15 min high-light stress^{+}, versus normal light†	Photosynthesis; and others
HL 1 h	1 h high-light stress^{+}, versus normal light†	Photosynthesis; and others
HLrec 2 h	2 h recovery^{+} after 1 h high-light stress†, versus before stress^{+}	Various functions
HLrec 48 h	48 h recovery^{+} after 1 h high-light stress†, versus before stress^{+}	Various functions
ML 4C	24 h medium light‡ at 4 °C, versus 20 °C	Photoinhibition of photosystems
+Par	Treatment with the benzoquinone herbicide paraquat, versus no treatment	Reactive oxygen species; photosynthesis; oxidative stress
+Bro	Treatment with the nitrile herbicide bromoxynil, versus no treatment	Photosynthesis; and others
–CO_2 2 d	Low-CO_2 stress (2 d at 0.003% (v/v) CO_2), versus normal CO_2 levels	Calvin cycle; respiration
–CO_2 4 d	Low-CO_2 stress (4 d at 0.003% (v/v) CO_2), versus normal CO_2 levels	Calvin cycle; respiration
+CO_2 1 d	High-CO_2 stress (1 d at 1% (v/v) CO_2), versus normal CO_2 levels	Calvin cycle; respiration
+CO_2 10 d	High-CO_2 stress (10 d at 1% (v/v) CO_2), versus normal CO_2 levels	Calvin cycle; respiration
+Fe	High-iron stress (spraying with iron solution¶), versus no treatment	Oxidative stress
+Pro	48 h with 100 mM proline, versus no treatment§	Increased levels of cellular proline
+CK	Cytokinin-treated (2 h) cell culture, versus untreated cell culture**	Various functions
Mutants		
prpl11	*prpl11-1* (Pesaresi *et al.*, 2001) versus WT	Lacks plastid ribosomal protein PRPL11; plastid protein synthesis
psad	*psad1-1*$^{++}$ versus WT	Lacks photosystem I protein PSI-D1 (photosynthesis)
psae	*psae1-1* (Varotto *et al.*, 2000) versus WT	Lacks photosystem I protein PSI-E1 (photosynthesis)
psan	*psan-1*$^{++}$ versus WT	Lacks photosystem I protein PSI-N (photosynthesis)
psao	*psao-1*$^{++}$ versus WT	Lacks photosystem I protein PSI-O (photosynthesis)
atpc	*atpc1-1*†† versus WT	Lacks ATP synthase subunit (photophosphorylation)
atpd	*atpd-1*$^{++}$ versus WT	Lacks ATP synthase subunit (photophosphorylation)
hcf145	*hcf145*†† versus WT	Assembly of photosystem I disrupted (chloroplast biogenesis)
gun1	*gun1-1* (Susek *et al.*, 1993) versus WT	Unknown; plastid signalling
gun5	*gun5* (Susek *et al.*, 1993; Mochizuki *et al.*, 2001) versus WT	ChlH subunit of magnesium chelatase (plastid signalling)
cue1	*cue1-1* (Streatfield *et al.*, 1999) versus WT	Lacks phosphoenol pyruvate translocator (metabolite exchange); plastid signalling
flu D	*flu* (Meskauskiene *et al.*, 2001; dark) versus WT (dark)	Lacks FLU, a regulator of chlorophyll biosynthesis
flu L	*flu* (Meskauskiene *et al.*, 2001; light) versus WT (dark)	Lacks FLU, a regulator of chlorophyll biosynthesis
ppi1	*ppi1* (Jarvis *et al.*, 1998) versus WT	Defective in import of chloroplast precursor proteins
kn09	*kno9*§ versus WT	Blocked in mitochondrial proline catabolism
sut2	*sut2*§ versus WT	Lacks the plasma-membrane sucrose-sensor/transporter (carbohydrate partitioning)
mak3	*atmak3-1*$^{++}$ versus WT	Defective in cytoplasmic *N*-acetyltransferase (various functions)
pam48	*pam48*$^{++}$ versus WT	Defective in unknown protein (altered photosynthesis)
pam46	*pam46*$^{++}$ versus WT	Defective in unknown protein (altered photosynthesis)

*RNA provided by T. Pfannschmidt; $^{+}$2,000 μmol photons m^{-2} s^{-1}; †80 μmol photons m^{-2} s^{-1}; ‡100 μmol photons m^{-2} s^{-1}, RNA provided by A. Haldrup; ¶leaves were sprayed with a 0.06% Fe^{2+}-chelate solution; §RNA provided by W. Frommer and co-workers; **provided by T. Schmülling; $^{++}$unpublished mutant isolated in our laboratory; ††provided by J. Meurer. WT, wild type.

responses, all 3 types were observed; however, most conditions resulted predominantly either in upregulation or in downregulation, whereas only 8 led to a mixed response (Fig. 1). Interestingly, most conditions tend to induce or repress transcription of the 631 non-chloroplast-protein genes in a similar way, suggesting that the regulation of chloroplast functions is integrated into larger regulatory networks.

Classification of transcriptomes

The analysis described above did not distinguish between whether similar or completely different sets of genes were co-regulated under different conditions. Therefore, the expression profiles of a subset consisting of 1,972 genes (including 1,542 that encode chloroplast proteins), which were selected because

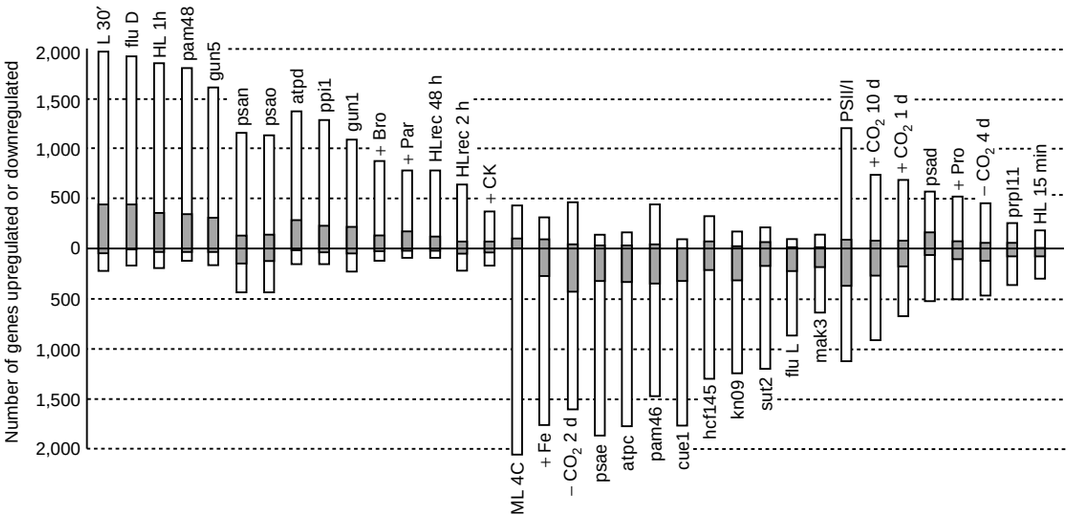

Fig. 1 | Effects of 35 environmental or genetic states on the nuclear chloroplast transcriptome. Three groups of differential transcriptome changes, including preferential upregulation, downregulation, or a mixed type of gene expression, are shown. The numbers of upregulated and downregulated genes are indicated by columns above and below the *x* axis. Unfilled areas indicate differential expression of nuclear chloroplast genes, and grey shading indicates genes that encode non-chloroplast proteins. Abbreviations are defined in Table 1.

they respond transcriptionally under at least 33 of the 35 conditions, were subjected to hierarchical clustering. From the results of this (Fig. 2), it was clear that sets consisting mostly of the same genes were co-ordinately upregulated or downregulated.

To characterize the similarities between the 35 transcriptome responses in more detail, hierarchical clustering was combined with an analysis that compares many gene-transcription interrelationships: pairwise comparisons of transcriptome responses were performed ('all-against-all'; see supplementary information online). The results of this combined analysis are shown as a circle diagram (Fig. 3), in which the 35 transcriptome responses are arranged in the order defined by hierarchical clustering (Fig. 2), and the many relationships between transcriptome reponses are indicated by connecting lines. A well-organized structure emerged, in which three main types of transcriptome response are apparent. Two correspond well to the classes already identified as involving predominantly upregulation (class 1) or downregulation (class 3), whereas class 2 is characterized by expression profiles showing some relationship to classes 1 and 3.

When only the 23 environmental or genetic perturbations that resulted in transcriptome responses belonging to classes 1 and 3 were considered, a set of 1,349 genes (including 1,022 that encode chloroplast proteins) was identified, the members of which were expressed differentially in all 23 conditions. Of these, 641 encode proteins with unknown functions. Among those with known functions, 116 are involved in transcription or translation, 135 in metabolism, 71 in photosynthesis, 46 in transport, and 53 encode protein kinases or phosphatases. Hierarchical clustering of the expression profiles for the 1,349 genes showed that almost all of the genes were affected in opposite ways in the two classes of transcriptome response: class 1 genes were upregulated and class 3 genes were downregulated (Fig. 4). Furthermore, detailed analysis of the expression profiles revealed that, within a particular class, additional levels of variation were present, with a few cases even showing reversed behaviour for a particular gene tested under specific conditions. This implies the existence of additional regulatory mechanisms that differentiate and modify the general responses of the nuclear chloroplast transcriptome that are induced by class 1 and 3 conditions. Indeed, some class 2 transcriptome responses (for example, in the *pam46* mutant; or with 1 h of high-light stress, followed by recovery for 48 h (HLrec 48h); Fig. 3), might result from such combined mechanisms.

Evidence for a master switch

The discovery of two major and opposite responses implies the existence of a two-state switch that integrates information about perturbations, and subsequent homeostatic adjustments, due to altered genetic and environmental states. This switch, once activated, causes either class-1-type or class-3-type expression changes. Indirect evidence for such a switch is provided by the opposite responses seen in two types of plastid signalling mutants, for which such a contrasting pattern of response might be expected: *gun1* and *gun5* mutants (Susek *et al.*, 1993; Mochizuki *et al*., 2001), and the *cue1* mutant (a member of a second class of plastid signalling mutants, the 'chlorophyll *a*/*b*-binding-protein-underexpressed' mutants; Streatfield *et al*., 1999). Almost all genes that were upregulated in *gun1* and *gun5* mutants were downregulated in *cue1* mutants (see supplementary information online). This is reflected in the hierarchical clustering analysis, where these mutations showed either class 1 or class 3 expression effects. The situation for the FLU protein is similar; dark-adapted plants with mutations in *FLU* (see flu D, Figs 3,4), which encodes a regulator of tetrapyrrole biosynthesis (Meskauskiene *et al*., 2001), showed a class-1 expression profile. When dark-adapted *FLU* mutants were exposed to light for 30 min (flu L, Figs 3,4), this was reversed to

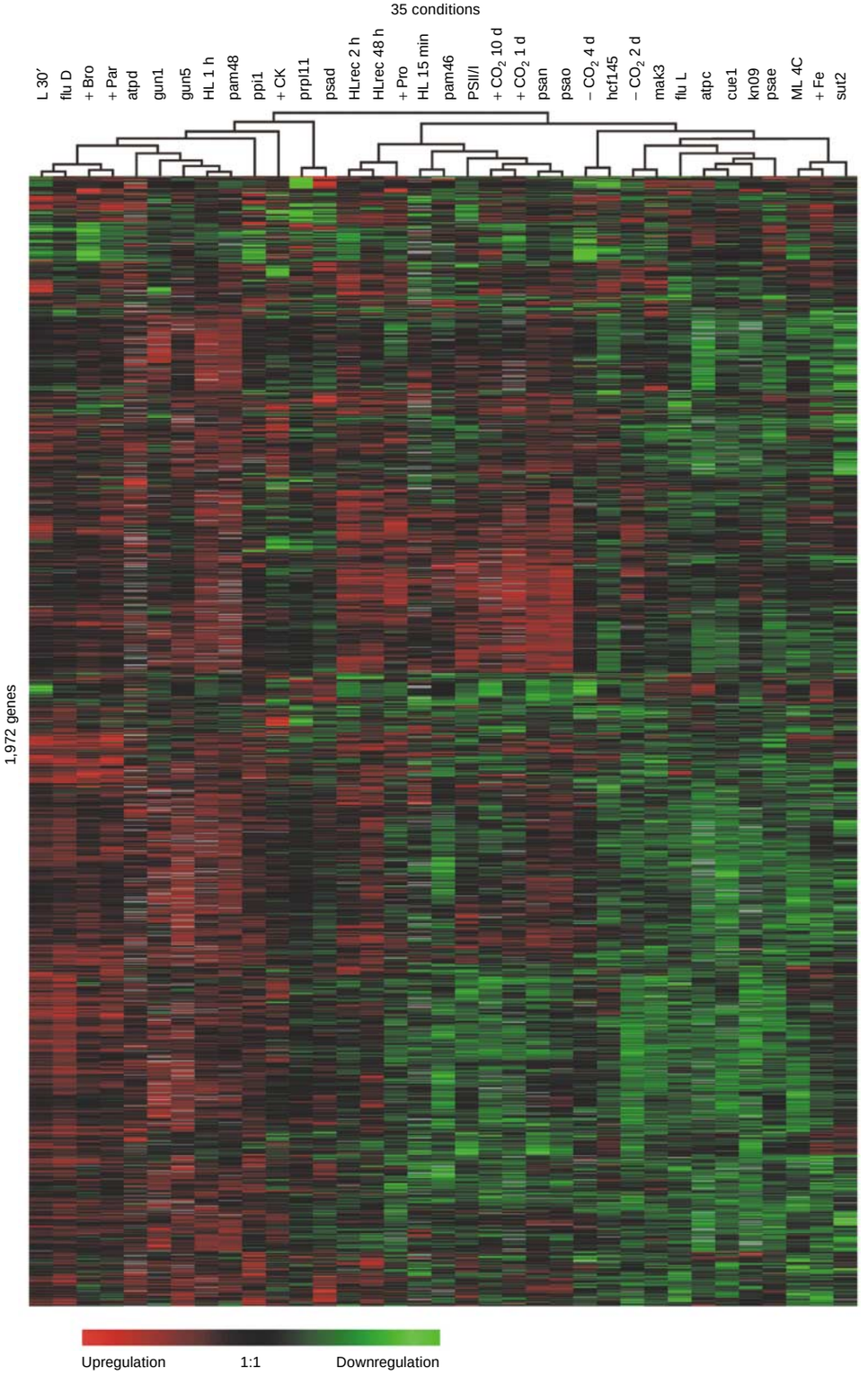

Fig. 2 | Hierarchical clustering of the expression profiles of 1,972 genes that show differential expression under at least 33 of the 35 conditions. The cladogram at the top summarizes the relatedness of transcriptome responses. Abbreviations are defined in Table 1. Colours indicate the upregulation (red) or downegulation (green) of genes, with respect to wild-type controls. Grey lines indicate missing values.

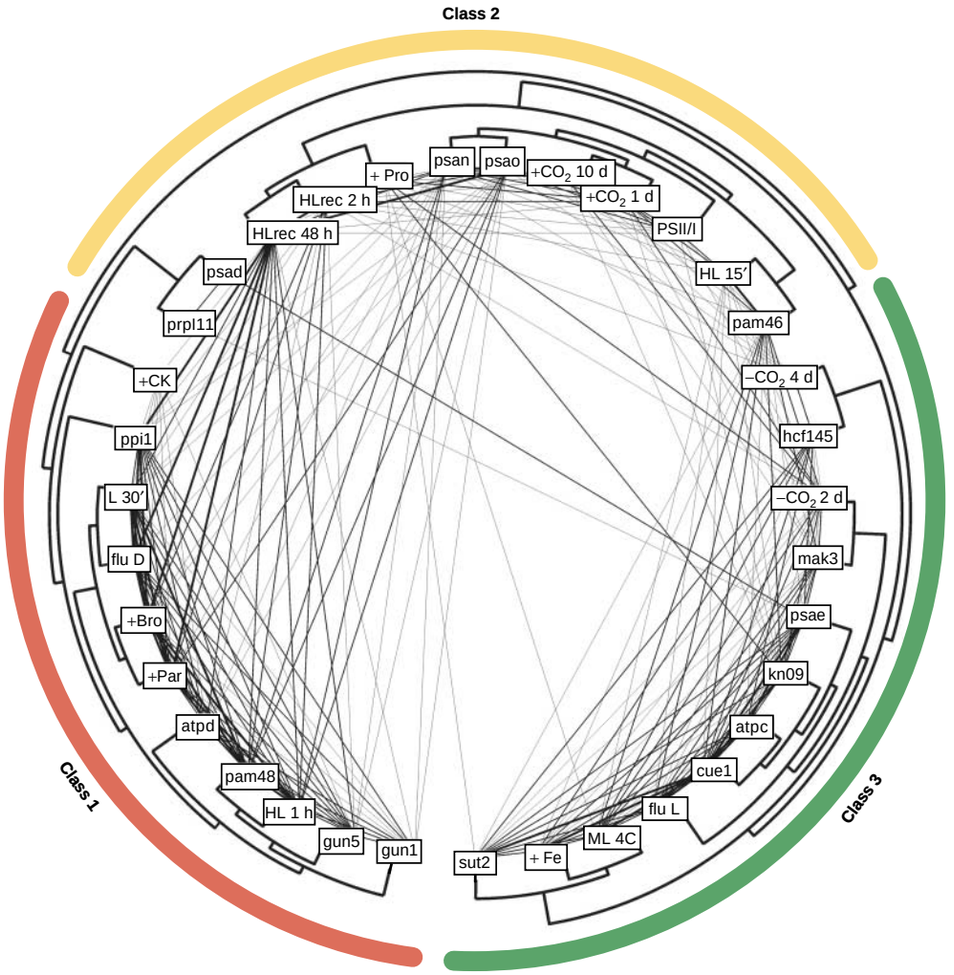

Fig. 3 | The 35 nuclear chloroplast transcriptome responses fall into three main classes. The topographical arrangement of environmental and genetic conditions is derived from Fig. 2, and the cladogram in the same figure is shown at the periphery. The relationships between expression profiles are indicated inside the circle by connecting lines. Increasing line thickness indicates increasing relatedness, with the three thicknesses of lines corresponding to 65–80%, 80–90% and 90–100% of genes showing the same trend in expression in each pair of transcriptomes. In some cases, cladogram branches were rotated, without changing the overall topography, to minimize the length of the connecting lines. Main classes of transcriptome change are indicated in red (class 1), yellow (class 2) and green (class 3).

give a class-3 expression profile. This, together with what is known about the regulatory role of FLU in chlorophyll biosynthesis (Meskauskiene *et al*., 2001), and the role of tetrapyrroles in GUN-type signalling (Strand *et al*., 2003), suggests that *FLU* may have a direct role in controlling signal stability.

Unexpectedly, some conditions, which, based on our present knowledge, should produce similar physiological perturbations, instead induced opposite responses. This includes the mutants *atpd-1* and *atpc1-1*, which, although they affect the same protein complex (ATPase), induce class-1 and class-3 responses, respectively. This implies that the two mutations cause different changes in ATPase function, resulting in discrete transcriptional responses. In fact, we saw a similar behaviour for *psae1-1*, *psad1-1*, *psan-1* and *psao-1*: these photosystem I mutants clearly differ, both in their photosynthetic phenotypes (data not shown) and their mRNA expression profiles (classes 1 or 2).

Mutation of *psae1-1* and high-iron treatment are two conditions that are thought to affect the cellular redox state (Varotto *et al*., 2000), or to increase the level of oxidative stress (Halliwell & Gutteridge, 1984). Both conditions lead to a class-3 response; conversely, herbicides or irradiation in the cold, two treatments that are also expected to increase the level of oxidative stress (Böger & Sandmann, 1998; Tjus *et al*., 2001), induce class-1 responses.

These unexpected responses provide strong evidence for the existence of a master switch that is independent of the type of physiological adjustment; in fact, perturbations in signalling (*gun5*, *cue1* and *flu* mutations), in the ATP:ADP ratio (*atpd-1* and *atpc1-1* mutations), or in the redox state (*psae1-1* mutants, herbicide treatments and photoinhibitory conditions), should result in different physiological states, but all of these states are translated into one of the two possible modes of action: upregulation or downregulation of, mainly, the same set of genes.

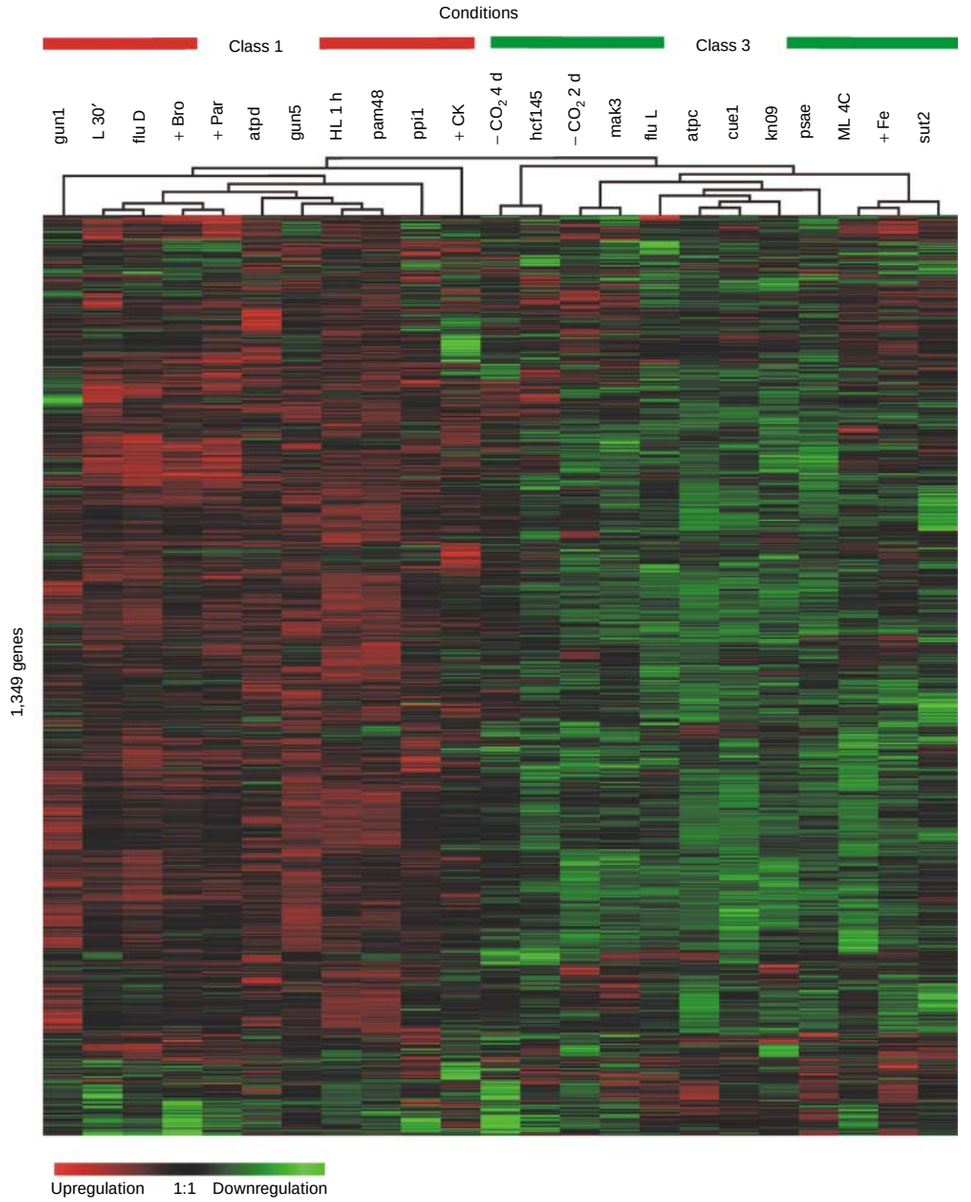

Fig. 4 | Class-1-type and class-3-type responses generally involve the same set of genes. 1,349 genes, which were differentially expressed in all of the 23 conditions that elicited upregulation (class-1 responses) or downregulation (class-3 responses), were clustered hierarchically, with red corresponding to upregulation and green to downregulation. Each gene is represented by a horizontal line, which, in most cases, shifts from red (left ; class-1 conditions) to green (right; class-3 conditions).

Other types of responses

Class-2 transcriptome changes fall into several subclasses (Fig. 3), and are induced by conditions such as a shift in light quality (PSII/I), high CO_2 concentrations (for 1 day or 10 days), or mutations of the photosynthetic machinery (*psad1-1*, *psan-1* and *psao-1*). All these genetic and environmental conditions have been reported to change the redox state and/or the concentration of reactive oxygen species in the chloroplast (Pfannschmidt *et al*., 2001; Mullineaux & Karpinski, 2002; Noctor *et al*., 2002). Thus, the observed responses, in terms of the regulation of the nuclear chloroplast transcriptome, indicate that information about the redox state or the concentration of reactive oxygen species feeds into a more complex signalling pathway, which induces some genes and represses others.

DISCUSSION

Chloroplast functions are regulated at several levels: chloroplast and leaf development is regulated by light signal-transduction, which involves phytochromes and cryptochromes. It is also known that the photosynthetic process itself provides signals for its own regulation, as well as that of other plastid and non-plastid

processes (Allen *et al*., 1995; Jarvis, 2001; Rodermel, 2001; Mullineaux & Karpinski, 2002). Intra-organellar signalling that senses the redox state of the photosynthetic apparatus modulates the expression of certain plastome genes (Pfannschmidt *et al*., 1999), and/or the activity of plastid protein-kinases (Forsberg *et al*., 2001). During the evolution of the plant lineage, a transfer of large numbers of chloroplast genes into the nucleus occurred (Martin & Herrmann, 1998; Martin *et al*., 2002). Therefore, inter-organellar signalling and co-ordinate expression of sets of nuclear genes had to be established (Jarvis, 2001). Previous studies have provided insights into the emitter component of plastid-to-nucleus signal relays (Surpin *et al*., 2002), whereas this study proposes the existence of a major switch that regulates the nuclear chloroplast transcriptome as a whole. The two main responses that were induced by most of the conditions tested, which included diverse and apparently unrelated conditions, are characterized by expression profiles typical of, or opposite to, those of *GUN*-type mutants that affect plastid signalling (Surpin *et al*., 2002; Strand *et al*., 2003). It therefore seems that GUN-type signalling is embedded in the larger type of regulatory network controlled by the master switch described here.

Co-ordinated expression of nuclear genes in response to different treatments has been described for prokaryotes and eukaryotes. Examples include the SOS response genes of *Escherichia coli* (of which there are at least 30), which show a co-ordinate induction of expression after treatments that lead to DNA damage (Sutton *et al*., 2000; Khil & Camerini-Otero, 2002), and the environmental stress response in yeast, in which 900 genes are activated on exposure to several stresses (Gasch *et al*., 2000). Here, we have shown that specific treatments, or genetic defects, result in three discrete types of co-ordinate transcriptional responses by nuclear genes that encode chloroplast proteins. This 'transcriptome-response map' provides a framework for the classification of novel chloroplast-function mutants and/or additional treatments based on their expression profiles. By increasing the number of conditions, additional mechanisms of transcriptional responses, acting in parallel or downstream of the three responses described here, should be unravelled.

METHODS

Plant material and growth conditions. *Arabidopsis* plants were propagated for 4 weeks under controlled greenhouse conditions, and treated as in Table 1, except that *hcf145*, *ppi1*, *atpc1-1* and *atpd-1* plants were propagated with control plants on Murashige–Skoog medium supplemented with sucrose. Total RNA from leaves was isolated 2 h after the beginning of the day period, in accordance with the methodology of Kurth *et al*. (2002).

Generation, testing and use of the 3,292-gene-sequence-tag array. The 1,827 GSTs described in Kurth *et al*. (2002) were combined with an additional 1,465 GSTs from genes that encode proteins that have a TargetP-predicted cTP (Emanuelsson *et al*., 2000). Proteins for which TargetP indicated only a low probability of their containing a cTP were excluded. Amplification, quantification, verification and spotting of PCR products was performed according to Varotto *et al*. (2001) and Kurth *et al*. (2002). Possible cross-hybridization among the 3,292 GSTs and the rest of the *Arabidopsis* genome was tested by BLAST analysis: only 14 GSTs contained stretches of >100 bp that matched another GST, and only 90 GSTs showed sequence homology to any other *Arabidopsis* ORF.

At least three experiments, with different filters and independent complementary DNA probes from plant pools, were performed for each condition, thus minimizing the variation between individual plants, filters or probes. cDNA-probe synthesis was primed using a mixture of oligonucleotides that matched the 3,292 genes in the antisense orientation, and were hybridized to the GST array as described in Kurth *et al*. (2002). Images were read using the Storm phosphoimager (Molecular Dynamics).

Data analysis. Hybridization images imported into ArrayVision (version 6.0; Imaging Research) were statistically evaluated using ArrayStat (version 1.0; Imaging Research). Data were normalized with reference to all spots on the array (Kurth *et al*., 2002), and average expression ratios derived from at least three independent experiments (for further information see http://www.mpiz-koeln.mpg.de/~leister/chloroplast_1.html) were clustered hierarchically using Genesis software (version 1.1.3; Sturn *et al*., 2002).

For pairwise 'all-against-all' comparisons, ratios of the number of genes that were differentially expressed in the same direction versus all genes differentially expressed in each pair of conditions were calculated. The resulting matrix was processed by the PhyloGrapher program (http://www.atgc.org/PhyloGrapher/).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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